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Vaccine-Induced Cellular Immune Responses Reduce Plasma Viral Concentrations after Repeated Low-Dose Challenge with Pathogenic Simian Immunodeficiency Virus SIVmac239

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The goal of an AIDS vaccine regimen designed to induce cellular immune responses should be to reduce the viral set point and preserve memory CD4 lymphocytes. Here we investigated whether vaccine-induced cellular immunity in the absence of any Env-specific antibodies can control viral replication following multiple low-dose challenges with the highly pathogenic SIVmac239 isolate. Eight *Mamu-A*01*-positive Indian rhesus macaques were vaccinated with simian immunodeficiency virus (SIV) *gag*, *tat*, *rev*, and *nef* using a DNA prime-adenovirus boost strategy. Peak viremia ($P = 0.007$) and the chronic phase set point ($P = 0.0192$) were significantly decreased in the vaccinated cohort, out to 1 year postinfection. Loss of $CD4^+$ memory populations was also ameliorated in vaccinated animals. Interestingly, only one of the eight vaccinees developed Env-specific neutralizing antibodies after infection. The control observed was significantly improved over that observed in animals vaccinated with SIV *gag* only. Vaccine-induced cellular immune responses can, therefore, exert a measure of control over replication of the AIDS virus in the complete absence of neutralizing antibody and give us hope that a vaccine designed to induce cellular immune responses might control viral replication.

The only long-term solution to the human immunodeficiency virus (HIV) epidemic in the developing world is likely to be a vaccine that either prevents infection or substantially reduces transmission. The failure of the AIDSVAX HIV vaccine was announced in 2003 (25), showing that Env-specific vaccine-induced antibodies that do not neutralize primary HIV type 1 will not prevent infection. Development of an HIV vaccine that induces broadly reactive neutralizing antibodies has proven to be difficult due to the enormous diversity of the envelope and the difficulty in neutralizing primary isolates. Much of the vaccine field is therefore currently focused on generating immunogens that induce potent cellular immune responses and control viral replication. This vaccine's aim would be to ameliorate disease course and reduce virus transmission. The risk of transmission is greatest at the times of highest viremia, that is, during acute infection and uncontrolled chronic infection. An HIV vaccine that induces cellular immune responses, therefore, should aim to limit peak viremia in acute infection and to reduce chronic-phase plasma viral concentrations from the median level of ~30,000 copies/ml in untreated patients, to levels at which transmission is unlikely. Infected individuals with viral loads of <1,700 copies/ml fail to transmit the virus to their HIV-negative partners (27, 28, 52). The WITS Study in 1999 (23) of over 550 mother-child pairs found that there was negligible risk of mother-to-child transmission when the maternal viral load was <1,000. Vaccine-mediated reduction of viral replication in chronically infected subjects to these levels would, therefore, reduce transmission, in addition to ameliorating disease course (43, 44, 59).

Many candidate vaccines have been evaluated in macaque models. Unfortunately, few vaccine regimens designed to induce cellular immune responses in the absence of Env-specific antibodies have significantly lowered plasma viral concentration or affected disease course in macaques using stringent simian immunodeficiency virus (SIV) challenge models. Recently, several vaccines have claimed success in control of the

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chimeric simian human immunodeficiency virus SHIV89.6P (3–5, 14, 39, 54, 57) virus. However, all but one of those vaccine regimens includes a closely matched Env in the vaccine, and serious doubts have surfaced as to the suitability of SHIV89.6P as a challenge model (20). The only regimen that did not use Env as an immunogen, a DNA prime-recombinant adenovirus boost encoding only Gag, was marginally effective against an SIV SIVmac239 challenge (11), despite its success against SHIV89.6P (57).

Several vaccine regimens have shown promise against the simian immunodeficiency viruses SIVmac251 (6, 8, 9, 17, 26, 30, 47, 48, 67), SIVsmE660 (18, 32, 33, 46) and SIV/DeltaB670 (21). However, most of these trials used an envelope immunogen with sequence similarity to the challenge virus (45). More recently, Hel et al. showed some efficacy using early proteins, in addition to an Envelope immunogen, to ameliorate the pathogenic effects of SIVmac251 infection (31), indicating the promise these early proteins offer. Three vaccine approaches, using Gag only as an immunogen (11, 19, 40), showed reduction of viral replication in some of the vaccinees, but showed no statistically significant reduction overall in chronic-phase viral replication between the vaccinees and controls. Here we tested whether a vaccine that encodes proteins designed to induce cellular immune responses, in the absence of envelope-specific antibodies, can reduce viral replication after challenge with the highly pathogenic SIVmac239 isolate.

Various investigators have suggested that cellular immune responses against proteins expressed early in the viral life cycle may have a leading role to play in protection against viral challenge. $CD8^+$ lymphocyte clones against Nef (64, 65) and Rev (60, 61) suppress viral replication in vitro. Indeed, movement of an reverse transcription (RT)-derived $CD8^+$ lymphocyte epitope from Pol to Nef resulted in increased susceptibility of cells infected with the recombinant virus to lysis by an RT-specific $CD8^+$ lymphocyte clone (60). These data and early vaccine work (22) suggest that $CD8^+$ lymphocytes against these early proteins play an important role in containment of viral replication. Here we induced cellular immune responses (in the absence of Envelope-specific antibodies) against Gag, Tat, Rev, and Nef and challenged macaques with the highly pathogenic SIVmac239 isolate.

MATERIALS AND METHODS

Animals and challenges. The animals in this study were Indian rhesus macaques (*Macaca mulatta*) from the Wisconsin National Primate Research Center colony. They were typed for major histocompatibility complex (MHC) class I alleles *Mamu-A*01*, *Mamu-A*02*, *Mamu-A*08*, *Mamu-A*11*, *Mamu-B*01*, *Mamu-B*03*, *Mamu-B*04*, *Mamu-B*17*, and *Mamu-B*29* by sequence-specific PCR (34; also data not shown). Animals that were *Mamu-A*01* positive were chosen for the study, but animals positive for *Mamu-B*17* were excluded. It has recently been observed that the presence of the *Mamu-B*17* allele alone is correlated with a reduction in plasma viremia (66). The animals were cared for according to the regulations and guidelines of the University of Wisconsin Institutional Animal Care and Use Committee. We used a low-dose challenge regimen (42) of SIVmac239 to more closely mimic HIV transmission in humans. Recent findings support the notion that multiple exposures are required to cause infection in humans (12, 28). More recently, it has been suggested that HIV transmission could be as high as 1:50 during the acute phase (15, 50, 63). We have previously shown that multiple low-dose challenges achieved infection after several doses, and further, after infection, the kinetics of viral replication were very similar to those caused by high-dose challenge. Animals were challenged intrarectally with 300 $TCID_{50}$ (tissue culture infectious dose sufficient to infect 50% of cells) of SIVmac239. Challenges were performed weekly until a blood draw at 7 days postchallenge indicated that they were infected.

Vaccine. Genes coding for SIVmac239 Gag, Tat, Rev, and Nef were synthesized based on codons frequently used in mammalian cells (57). The *nef* gene encoded a full-length protein with a mutation at the myristoylation site (G2A) for inactivation. The genes were cloned into the V1R vector as previously described (57). Five milligrams of DNA was added to 7.5 mg of CRL1005 (CytRx) mixed with benzalkonium chloride (Ruger). Before the intramuscular immunization of 0.5 ml of this vaccine, the formulation was warmed slowly to room temperature from a frozen stock. The same codon-optimized genes were cloned into an adenoviral vector based on serotype 5 adenovirus that had been rendered incompetent to replicate by deletions of the E1 viral gene and subsequently propagated in E1-expressing PER.C6 cells, as previously described (57). Virus particles (10^{11}) were injected intramuscularly. Vaccines were administered into four separate sites, a different site for each encoded protein to prevent immunodominant suppression of subdominant responses.

Viral load determination. The plasma virus concentration was determined using a modification of a protocol published by Lifson et al. (36, 37). Briefly, viral RNA was isolated from plasma and amplified in a quantitative single-step RT-PCR using the Roche Master Probes kit, with reactions performed in the Roche LightCycler 2.0. Primers and *Taq*Man probes were designed according to the sequences published by Lifson et al., except that our primer and probe sequences exactly matched the SIVmac239 sequence. The quantity of viral RNA copies initially present was determined by extrapolation of threshold fluorescence values onto an internal standard curve prepared from serial dilutions of an in vitro-transcribed fragment of the SIVmac239 gag gene (vector kindly provided by M. Piatak and J. Lifson).

Intracellular cytokine staining assay. The intracellular cytokine staining assay was performed essentially as described in detail previously (62). Briefly, 500,000 freshly isolated peripheral blood mononuclear cells (PBMC) were incubated for 1.5 h at 37°C in 200 μl of R10 (RPMI 1640 containing 10% fetal calf serum and antibiotics) with anti-CD28, anti-CD49d, and synthetic peptides (pools of 15-mers or single peptides representing minimal optimal cytotoxic T-lymphocyte epitopes) based on the wild-type protein sequence. Then 10 μg of brefeldin A per ml was added to prevent protein transport from the Golgi apparatus, and the cells were incubated a further 5 h at 37°C. Cells were washed and stained for surface expression of CD4 and CD8 markers and fixed overnight in 1% paraformaldehyde at 4°C. The following day, cells were permeabilized in buffer containing 1% saponin and stained for expression of the cytokines gamma interferon (IFN-γ) and interleukin-2 (IL-2) before being fixed in 1% paraformaldehyde for 2 h at 4°C. Events were collected on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.) with CellQuest software and analyzed with FlowJo v.6.1.1 or above for Macintosh (Treestar, Ashland, Oreg.). A positive result is defined as being at least twofold higher than the negative control, preferably with the mean fluorescent intensity at least in the second decade above the negative peak.

MHC class I tetramer assay. We stained 500,000 cells (fresh PBMC or bulk cytotoxic T-lymphocyte cultures) with 2 μg of tetramers (labeled with phycoerythrin or allophycocyanin) for 1 h at 37°C in a volume of 200 μl of R10. Then 3 μl of anti-human CD3-fluorescein isothiocyanate conjugate (BD Pharmingen, San Diego, Calif.) and 6 μl of anti-CD8-peridinin chlorophyll protein (PerCP) (Becton Dickinson) were added, and the mixture was incubated for a further 45 min at room temperature. Cells were then washed twice in R10 or flow cytometry buffer (phosphate-buffered saline containing 2% fetal bovine serum and 1% bovine serum albumin) and fixed for at least 30 min at 4°C in 1% paraformaldehyde. Flow cytometry data were collected and analyzed as above.

ELISPOT assay. PBMC were used directly in IFN-γ or IL-4 enzyme-linked immunospot (ELISPOT) assays as previously described (1). Briefly, either pools of 9-mer, 10-mer, or 15-mer peptide from all proteins were added to 1.0×10^5 PBMC per well and incubated for 16 to 18 h at 37°C in a 5% CO_2 incubator. In addition, minimal optimal epitopes for *Mamu-A*01* were tested, as were minimal optimal epitopes for *Mamu-A*02* in macaques expressing this allele (38). All tests were performed in duplicate. Wells were imaged with an ELISPOT reader (AID, Strassberg, Germany), counted by EliSpot Reader, version 3.1.1 (AID, Strassberg, Germany), and analyzed as previously described (38). Spots were counted by an automated system with set parameters for size, intensity, and gradient. Background (mean of wells without peptide) levels were subtracted from each well on the plate. A response was considered positive if the mean number of spot-forming cells (SFCs) of duplicate sample wells exceeded background plus two standard deviations and was >50 SFC per 1×10^6 cells.

Memory markers. Analysis of CD4 memory populations was performed basically as described previously (49, 51). Briefly, PBMC were isolated from EDTA anticoagulated blood and stained for 7-parameter flow cytometry using the

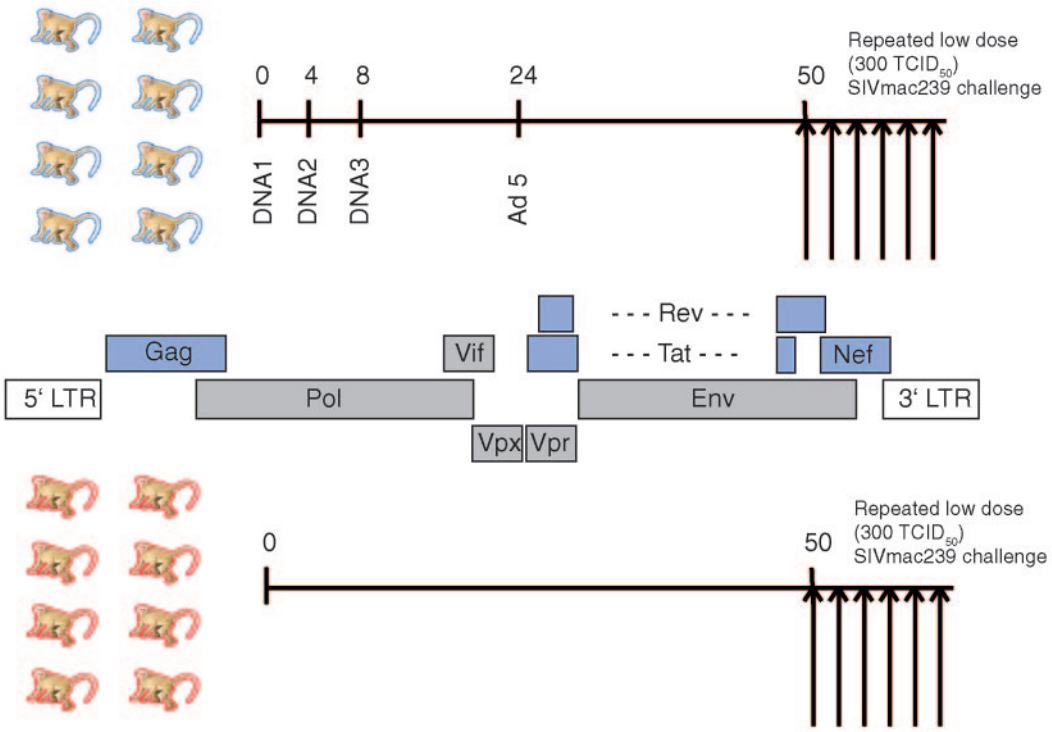

FIG. 1. Experimental design. Eight *Mamu-A*01*$^+$ Indian rhesus macaques (blue outline) were given DNA plasmid encoding Gag, Tat, Rev, and Nef added to the CRL1005/benzalkonium chloride adjuvant on weeks 0, 4, and 8. Each construct was given intramuscularly into a separate limb to minimize immunodominant suppression of a broad immune response. The same limb was used for the same encoded protein on each occasion. At week 24, Ad5 vectors, encoding the same four proteins, were given intramuscularly into the same limb as that used for the DNA primes. At week 50, the 8 vaccinees and 8 naive controls (red outline) were challenged with a low dose (300 $TCID_{50}$) of SIVmac239 (arrows) until a day 7 blood draw indicated that the animal had a positive plasma viremia by quantitative PCR. LTR, long terminal repeat.

following monoclonal antibodies: SP34 (CD3 phycoerythrin Cy7), L200 (CD4, PerCP), SK1 (CD8, PerCP), CD28.2 (CD28, phycoerythrin), DX2 (CD95, fluorescein isothiocyanate), and 3A9 (CCR5, allophycocyanin). Data were collected on an LSR II with the HeNe, Violet, and Sapphire lasers, and data were analyzed using FlowJo v6.1.1 or above for Macintosh (Treestar, Ashland, OR).

Statistics. Each variable was transformed, if needed, such that the model residuals were nearly normally distributed by using the Box-Cox family of transformations (7). We then tested for the differences via both parametric and nonparametric tests using two-tailed *P* values. For the chronic-phase set point, inspection of model residuals indicates marked nonnormality. Log transformation was taken and found to produce the greatest fit to normality. On the transformed scale, nonparametric analysis confirmed this result.

Neutralizing antibody assay. 293T cells grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin, streptomycin, L-glutamine, and fetal bovine serum (10%) were transiently transfected with DNA containing the SIVmac239 envelope along with a plasmid containing HIV structural genes and luciferase, using the calcium phosphate method. At 24 h posttransfection, the culture supernatants of all transfected cell cultures were replaced with fresh medium, and the cultures were incubated for a further 24 h. The supernatants containing pseudoviruses were harvested and either used fresh or, in some cases, frozen at −80°C until further use. One hundred microliters of pseudovirions (roughly generating between 10^5 and 10^6 relative light units) was mixed with various concentrations of antibody, incubated for 1 h at 37°C, added to cells, and incubated for a further 3 days. The luciferase activity from triplicate wells was measured on a luminometer (EG&G Berthold LB 96V; Perkin-Elmer, Gaithersburg, Md.), with the luciferase assay reagent (Promega), according to the manufacturer's instructions.

RESULTS

Vaccination with Gag, Tat, Rev, and Nef induces strong immune responses. Proteins expressed early in the SIV life cycle represent attractive targets for the immune system, since they may be recognized earlier, perhaps resulting in destruction of the infected cell prior to viral production. Therefore, genes encoding Tat, Rev, and Nef were incorporated into our vaccine constructs. Eight *Mamu-A*01*$^+$ Indian rhesus macaques were vaccinated with vectors expressing Gag, Tat, Rev, and Nef using three DNA immunizations and a single Ad5 boost (Fig. 1). Strong, broad vaccine-induced immune responses were seen in all vaccinees (Table 1), as evaluated by tetramer staining, ELISPOT, and intracellular cytokine staining (ICS), in both $CD8^+$ and $CD4^+$ compartments. Lymphocytes from vaccinated macaques made responses against an average of 9.1 $CD8^+$ epitopes and 11.8 $CD4^+$ epitopes.

Vaccine-induced cellular immune responses control viral replication. We used a low-dose repeated challenge regimen to determine the role of the vaccine-induced immune responses in both preventing infection and controlling viral replication after infection (42). Approximately 5 intrarectal (i.r.) challenges of 300 $TCID_{50}$ of SIVmac239 were required (Table 2) to infect both controls and vaccinees, indicating that vaccine-induced cellular immune responses cannot prevent infection. Interestingly, 1 vaccinee and 2 controls had multiple spikes of low-level transient viremia (data not shown). Since we could not rule out the possibility that our real-time PCR assay had resulted in a false positive, these macaques were excluded from further analysis.

Vaccine-induced anamnestic cellular immune responses were

TABLE 1. Tetramer staining, ELISPOT analysis, and ICS after vaccination with the DNA prime-adenovirus 5 boost regimen[a]

Animal identification no.	% of $CD8^+$ $CD3^+$ cells positive for tetramer		ELISPOT (10^6 SFC) result for:				Epitope breadth			
	CM9	SL8	Gag	Tat	Rev	Nef	$CD8^+$	$CD4^+$	$CD4^+$ IL-4^+	$CD4^+$ IFN-γ^+
00014	2.35	0.22	448	43	50	34	9	11	3	9
00044	23.4	1.22	3,698	615	1,905	1,945[b]	8	5	1	4
00060	15.8	1.56	3,365	386	324	321	11	2	1	1
01008	18.4	0.34	3,128	360	153	619[b]	7	33	1	32
96133	6.28	0.29	1,098	398	190	115	8	14	1	14
97111	2.41	0.6	1,485	310	38	194	14	14	10	11
97113	3.24	0.63	1,550	486	103	21	6	7	1	7
01080	0.7	0.34	478	400	359	451	10	8	0	8

[a] Vaccination with the DNA prime-adenovirus 5 boost regimen engenders strong, broad immune responses to all immunogens. Responses are shown for tetramer staining, ELISPOT analysis, and ICS. The immunogens used were pools of peptides (15-mers overlapping by 11 and 10-mers overlapping by 9) or individual peptides (15-mers, 10-mers, or minimal optimal peptides). To ascertain total breadth of response, we summed ICS, ELISPOT, and tetramer unique responses in either the $CD8^+$ (IFN-γ ELISPOT, ICS, and tetramer) or $CD4^+$ (ICS and IL-4 ELISPOT) subsets; this is presented in the last two columns of the table. For example, if a CM9 tetramer response was present as well as a response to the Gag E pool (which contains two peptides encoding CM9 within them) and the minimal optimal for CM9 in multiple tests, this would count as only one addition to the epitope breadth. Our goal was to ascertain the number of unique responses, not how many times we tested it. Tetramer responses are presented as percentages of tetramers positive in $CD3^+$ $CD8^+$ lymphocyte gated cells. $CD4^+$ responses were assessed at day 7 and day 14 post-adenovirus vaccination. Tetramer responses and ELISPOT responses were assessed throughout the postvaccination period; we only count the number of unique responses. CD4 responses are broken down into IL-4^+ and IFN-γ^+. Some responses are both IL-4^+ and IFN-γ^+.

[b] This animal is Mamu-A*02^+.

massive (Fig. 2) and reduced both viral peak and chronic-phase set point (Fig. 3). We calculated the chronic-phase set point over the entire length of the chronic phase (beginning at day 56 postinfection through 1 year postinfection) for each cohort.

TABLE 2. Vaccine-induced cellular immune responses do not affect the number of low-dose intrarectal challenges required for infection with SIVmac239[a]

Vaccinated or control animal	No. of challenges
Vaccinees	
r00014	3
r00044	6
r00060	7
r01008	6
r96133	4
r97111	2
r97113	7
r01080	4
Avg	4.88
Geometric mean	4.50
Controls	
r98059	7
r99034	2
r99084	3
r00041	8
r00045	3
r00021	3
r01034	5
r98050	6
Avg	4.63
Geometric mean	4.17

[a] Animals were challenged intrarectally with 300 $TCID_{50}$ of SIVmac239. One week later, plasma was analyzed for viral concentration, and the animal was rechallenged while awaiting the results of the viral concentration determination. The date of infection was considered to be 7 days prior to the first positive viral concentration, and the number of challenges was considered to be how many had occurred, including the one on this date.

Control macaques had peak plasma viral concentrations with a geometric mean of 5×10^7 copies/ml and progressed to late-chronic-phase set points of 149,000 copies/ml (Fig. 3b and c). In contrast, the cohort geometric mean of the peak viral loads for the vaccines is 1 log lower at less than 4×10^6 copies/ml. The vaccinee cohort had a late-chronic-phase set point geometric mean of less than 5,300 copies/ml, a reduction of 1.45 logs relative to that of the control cohort. Indeed, three vaccinees had chronic-phase plasma virus concentrations of less than 1,000 copies/ml at 1 year postinfection (Fig. 3a). Comparisons between the two groups of macaques revealed statistically significant differences between both peak ($P = 0.007$) and chronic-phase plasma viral concentration ($P = 0.0192$).

We observed a notable rank correlation between the magnitude of ELISPOT responses elicited by Rev (−0.75) during the vaccination phase and, to a lesser extent, the combined responses elicited by Tat plus Rev (−0.71) and peak viremia. These had a one-tailed P value of 0.033 and 0.04, respectively. We also observed a rank correlation between responses elicited to Tat (−0.75) and also Tat plus Rev (−0.71) and the chronic-phase viral concentration, with one-tailed P values of 0.033 and 0.04, respectively. While these are only suggestive of a trend and not statistically significant, the indication is that Rev and Tat responses appear to be involved in the additional level of control we observe compared to Gag-only vaccination (11). Doing the same analysis for $GagCM9_{181\text{-}189}$, $TatSL8_{28\text{-}35}$, Gag, and Nef, as well as combinations thereof, showed no correlation between these responses and either peak or chronic viral concentration.

A previous study using a DNA prime-Ad5 boost with Gag showed only transient control of SIVmac239 replication (11). We, therefore, compared the plasma viral concentrations observed in that study to the results of our current vaccine regimen (Fig. 3d). After approximately 80 days, vaccinees in the earlier study lost control of viral replication and there was no statistical difference between the control and vaccinated animals. While the plasma viral concentrations for control ani-

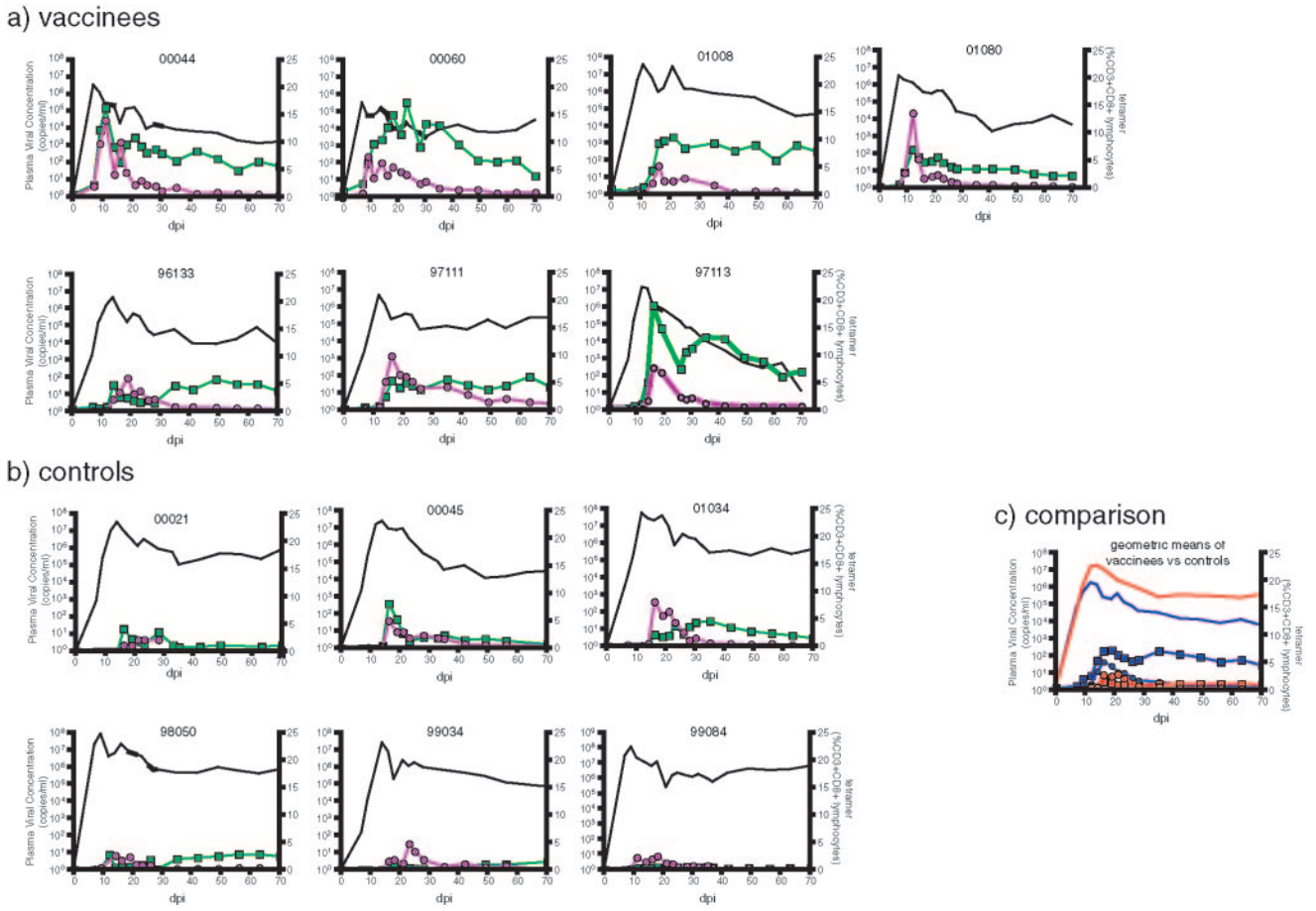

FIG. 2. Massive anamnestic $CD8^+$ lymphocyte responses were measured by tetramer staining after challenge with SIVmac239. Mamu A*01 Gag $CM9_{181–189}$ (□) and Mamu A*01 Tat $SL8_{28–35}$ (○) tetramers were used to stain PBMC. Tetramer-positive populations are expressed as percentages of $CD3^+$ $CD8^+$ lymphocytes. Plasma viral concentrations are shown as solid black lines. The figure also includes a geometric mean analysis of each cohort. Clearly, the vaccinees had stronger and earlier tetramer-positive populations to both Gag $CM9_{181–189}$ and Tat $SL8_{28–35}$, as seen in the comparison figure. Summary figures are in blue for vaccinees and red for controls.

mals in the previous study appear to be slightly higher, direct comparison of plasma virus concentrations in those control animals to our current control animal plasma samples indicates that there was no statistical difference between these groups ($P = 0.9151$). Addition of the regulatory proteins Tat, Rev, and Nef to the vaccine, therefore, facilitates control of plasma viral concentrations in the late chronic phase.

Vaccinees preserve critical CD4 memory populations. We analyzed fresh PBMC as well as longitudinal frozen samples for CD4 memory populations in the vaccinated and control macaques. At day 140 to 180, the vaccinees had significantly ($P = 0.0448$) ameliorated the loss of $CD4^+$ effector memory populations ($CD95^+$ $CD28^-$) compared to controls (Fig. 4a; Fig. 5a and b). Examination of $CCR5^+$ memory cells (CD3+ CD4+CD95+CCR5+ lymphocytes) in fresh PBMC demonstrated a similar level of preservation ($P = 0.0195$) (Fig. 4b, Fig. 5c), as did $CD3^+$ $CD4^+$ $CD95^+$ memory cells ($P = 0.0145$) (Fig. 4c; Fig. 5b and c). Indeed, overall $CD4^+$ lymphocyte populations were significantly higher in the vaccinees ($P = 0.0025$) (Fig. 4d). We also analyzed the memory CD4 compartment longitudinally, utilizing frozen cell samples preserved at several times prior to and after infection. The control animals experienced a dramatic acute-phase loss of $CCR5^+$ and effector memory cells. Interestingly, these populations increased in the vaccinees at 2 weeks postinfection (Fig. 4e). Normalized absolute CD4 counts over time make it clear that, while loss in the CD4 population is ameliorated in vaccines from the earliest phases of the experiment ($P = 0.0092$ from day 0 [d0] to d196), in the very late chronic phase (d203 to d336), vaccinees begin to recover these populations, while in controls they remain in a slow decline ($P = 0.0062$ from d203 to d336) (Fig. 4e, bottom panel). Therefore, at 1 year postinfection, some vaccinees are approaching CD4 counts comparable to those observed prior to infection. Taken together, our cellular immune response-based vaccine ameliorated memory CD4 loss.

Control of viral replication observed in vaccinees is not due to neutralizing antibodies. Since we did not vaccinate with Envelope, it is unlikely that we had induced neutralizing antibodies prior to challenge. However, it is possible that Env-specific neutralizing antibodies might develop after challenge and that these antibodies might be responsible for controlling viral replication in the vaccinees. We, therefore, measured neutralizing antibodies prior to vaccination, prior to challenge, and at 170 to 210 days postinfection using a pseudotype virus

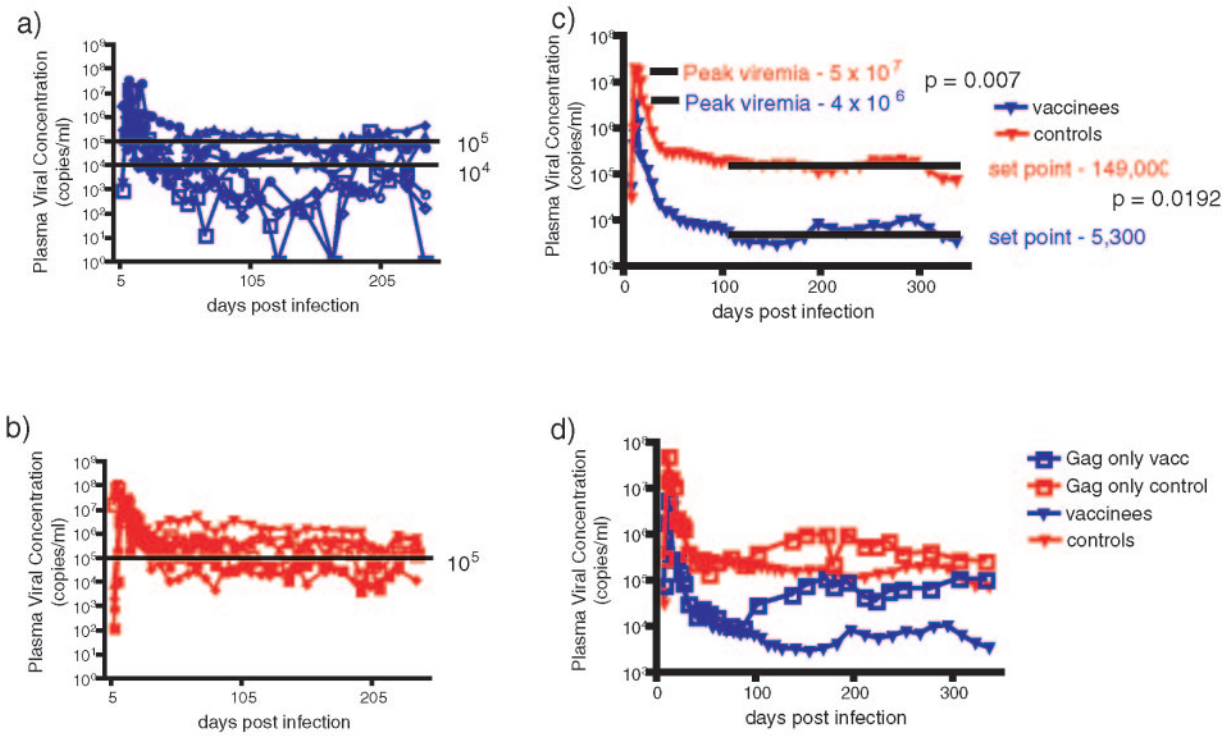

FIG. 3. Plasma viral concentrations are lower in vaccinees. Plasma viral concentration determinations were carried out by quantitative RT-PCR of plasma samples. (a) The majority of the vaccinees (in blue) had plasma viral concentrations of less than 10^5 copies/ml, including several animals whose plasma viral concentrations were less than 10^4 copies/ml, and at times, approached the limits of detection (open symbols). (b) However, control animals (in red) had chronic-phase plasma viral concentrations greater than 10^5 copies/ml. (c) Vaccinees had a significant reduction in peak viremia and chronic-phase set point. Taking a geometric mean for each group, we observed a significant ($P = 0.007$) reduction in peak viremia, from 5×10^7 on day 14 for control animals to 4×10^6 on day 11 for vaccinees. Chronic-phase viremia was also significantly decreased ($P = 0.0192$), from 1.49×10^5 in control animals, to 5,300 copies/ml in vaccinees. This is a reduction of plasma viral concentration of nearly 1.5 logs. (d) Compared to plasma viral concentrations for the Gag-only vaccine study (11), by 80 days postinfection, viral loads in the Gag-only study are increasing, whereas in the current study, control of plasma viremia is maintained well into the late chronic phase.

assay (68). Only one vaccinee developed neutralizing antibodies (animal 97111), the vaccinee with the poorest control of viral replication (Fig. 6). In contrast, all control animals except 98050 developed neutralizing antibodies after challenge (Fig. 7). Neutralizing antibodies against SIVmac239 can therefore be detected to SIVmac239 by our sensitive pseudotype virion assay in the controls. However, they do not develop in vaccinees, suggesting that control of viral replication in our vaccinees is completely independent of neutralizing antibodies.

DISCUSSION

The primary goal for a cellular immune response-based vaccine in humans is to reduce chronic-phase viremia enough to extend the time between infection and AIDS. A secondary goal should be to reduce viral transmission to uninfected contacts of the infected vaccinee. A third goal of such a cellular immune response-based vaccine would be to ameliorate the loss of memory $CD4^+$ cells that takes place in the acute phase (35, 41). One hallmark of long-term nonprogression in adults and children is preservation of the $CD4^+$ memory compartment (13, 55). Reduction from 30,000 copies/ml to less than 1,000 copies/ml, a 1.5 log reduction, prevents transmission of HIV (27, 28, 52) and increases longevity (43, 44, 59). In Indian rhesus macaques, the usual viral set point for SIVmac239 is between 10^5 and 10^6 copies/ml, so a 1.5 log reduction would result in a plasma viral concentration of approximately 3,000 to 30,000 copies/ml.

Very few vaccine regimens have influenced disease course after challenge with the highly pathogenic SIVs. By contrast, a variety of vaccines have induced immune responses that have controlled chronic-phase viral replication of SHIV89.6P (3–5, 14, 20, 39, 57). Unfortunately, many of these vaccines included an envelope with strong sequence similarity to the challenge virus. It is unlikely that the envelope of the vaccine and the envelope of the challenge virus will be similar in natural exposure to HIV type 1, given the sequence diversity of the envelope glycoprotein (10, 24). Broadly neutralizing antibodies will be required to neutralize the natural diversity of envelope sequences. These have been difficult to generate. In the absence of neutralizing antibody vaccines, we need to determine whether disease course can be ameliorated using T-cell responses alone. Vaccines that induce cellular immune responses are unlikely to provide sterilizing immunity because viral replication has to take place before cellular immune responses can be effective. However, it is possible that vaccine-induced cellular immune responses might control replication in the chronic phase. This would benefit both the health of the vaccinee and prevent transmission of the virus. Here we provide the first evidence that a vaccine that induces solely

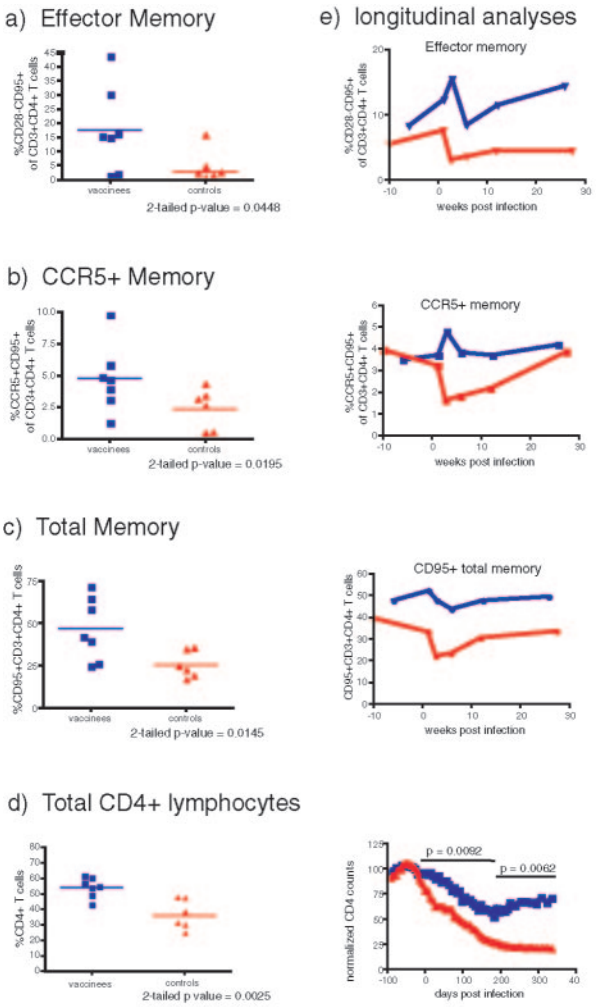

FIG. 4. Loss of $CD4^+$ memory populations is reduced in vaccinees at 140 to 180 days postinfection. PBMC were stained with antibodies to CD3, CD4, CD28, CCR5, and CD95 and read on an LSR II flow cytometer (see Fig. 5 for gating analysis). (a) The effector memory subset is significantly ($P = 0.0448$) preserved in vaccinees (blue) compared to controls (red). (b) The $CCR5^+$ population is also preserved in vaccinees ($P = 0.0195$). (c) Overall $CD95^+$ $CD4^+$ memory is preserved in vaccinees compared to controls ($P = 0.0145$). (d) Overall, the $CD4^+$ compartment is more robust in vaccinees than controls ($P = 0.0025$). (e) Analysis of longitudinal samples from frozen PBMC reveals that there is a drop in memory populations in control animals (red) during the acute phase, whereas some subsets actually increase in vaccinees (blue) during immediate acute infection. This is seen in the effector subset as well as the $CCR5^+$ subset. At week 2, rather than a depletion of this subset, there is actually an increase in these two populations in the vaccinated animals, while in the control animals, there is a decrease in these populations at this time point. The $CD95^+$ population is slightly higher in vaccinees at week 1 postinfection, likely because memory cells were engendered by the vaccination and the anamnestic response is already beginning to expand this population. By 25 weeks postinfection, the $CCR5^+$ subset is recovered to prechallenge levels in both control and vaccinated animals. However, the effector memory populations are still diverging, and the vaccinated animals have a net gain in this population. Normalized $CD4^+$ absolute counts are protected throughout the infection, both during the acute and early chronic phase (day 0 to 196; $P = 0.0092$) as well as during late chronic phase (day 203 to 336; $P = 0.0062$), during which time vaccinees are recovering their $CD4^+$ absolute counts, moving toward prechallenge values.

cellular immune responses can control chronic-phase viral replication.

Design of this vaccine regimen was based on a study that showed some control of chronic-phase replication of the highly pathogenic SIVmac239 challenge (11). In this previous study, *Mamu-A*01*$^+$ macaques were vaccinated with vectors encoding *gag* using a DNA prime-Ad5 boost regimen, then challenged with a high dose of SIVmac239. Similarly, a DNA prime-Sendai virus boost encoding *gag* resulted in control of viral replication in 5 of 8 Burmese macaques challenged with SIVmac239 (40). Evidence suggested an MHC-based control in the 5 successful vaccinees. We decided to build on these two encouraging studies by adding the early expressed genes of SIV to our vaccine regimen. We postulated that Tat-, Rev-, and Nef-specific $CD8^+$ effector lymphocytes would be able to kill virally infected target cells earlier in the viral life cycle than Gag-specific $CD8^+$ effector lymphocytes would, potentially prior to release of mature virus (29, 58, 60). Furthermore, since Nef downregulates MHC class I molecules (16), it is possible that $CD8^+$ effector lymphocytes directed against Tat, Rev, and Nef might be more effective, since these proteins may be expressed before MHC class I molecules are downregulated. In the vaccine, the *nef* antigen has been inactivated by incorporating a G2A mutation at the site of myristoylation to avoid this downregulation, which could decrease the effectiveness of the vaccine. Several investigators have made these arguments (2, 16, 56), but the hypothesis has not been rigorously tested with a stringent viral challenge. Recently, Hel et al. showed some efficacy using early proteins, along with Gag, Pol, and Envelope, to ameliorate the pathological effects of SIVmac251 infection (31), observing both decreased viremia and increased protection of $CD4^+$ responses to Gag in particular.

Peak viremia and early-acute-phase viral concentrations were comparable in vaccinees between the *gag*-only study and this study until about 80 days postinfection (Fig. 3d). After 80 days postinfection, the plasma viral concentration in vaccinees in the *gag*-only study began to rise, eventually becoming indistinguishable from the control group. By comparison, in the current study, the plasma viral concentrations in the vaccinees have remained low for a year postinfection.

It is unclear which cellular immune responses are actually responsible for control of viral replication in our vaccinees. We know that Env-specific antibodies played no role in the control of viral replication in the vaccinees in the initial stages of infection, since Env was not included as an immunogen and there are no neutralizing antibodies prior to challenge. Since the $CD4^+$ memory compartment shows some sign of preservation, it could be argued that Env-specific antibodies might have played a role in control later on in infection. Analysis of neutralizing antibodies, however, suggests that this was not the case. Enzyme-linked immunosorbent assay and Western blot analyses confirmed that all animals made antibodies to structural proteins, including Env (data not shown). However, the vaccinees did not make neutralizing antibodies, with the exception of 97111, who did not control her viral loads. Precedence for this result is found in the lymphocytic choriomeningitis virus system, where the presence of CD4 T helper responses prevented or delayed development of neutralizing antibody (53). In fact, the animals that exerted the best control of viral replication (97113, 00044, and 01080), and also exhib-

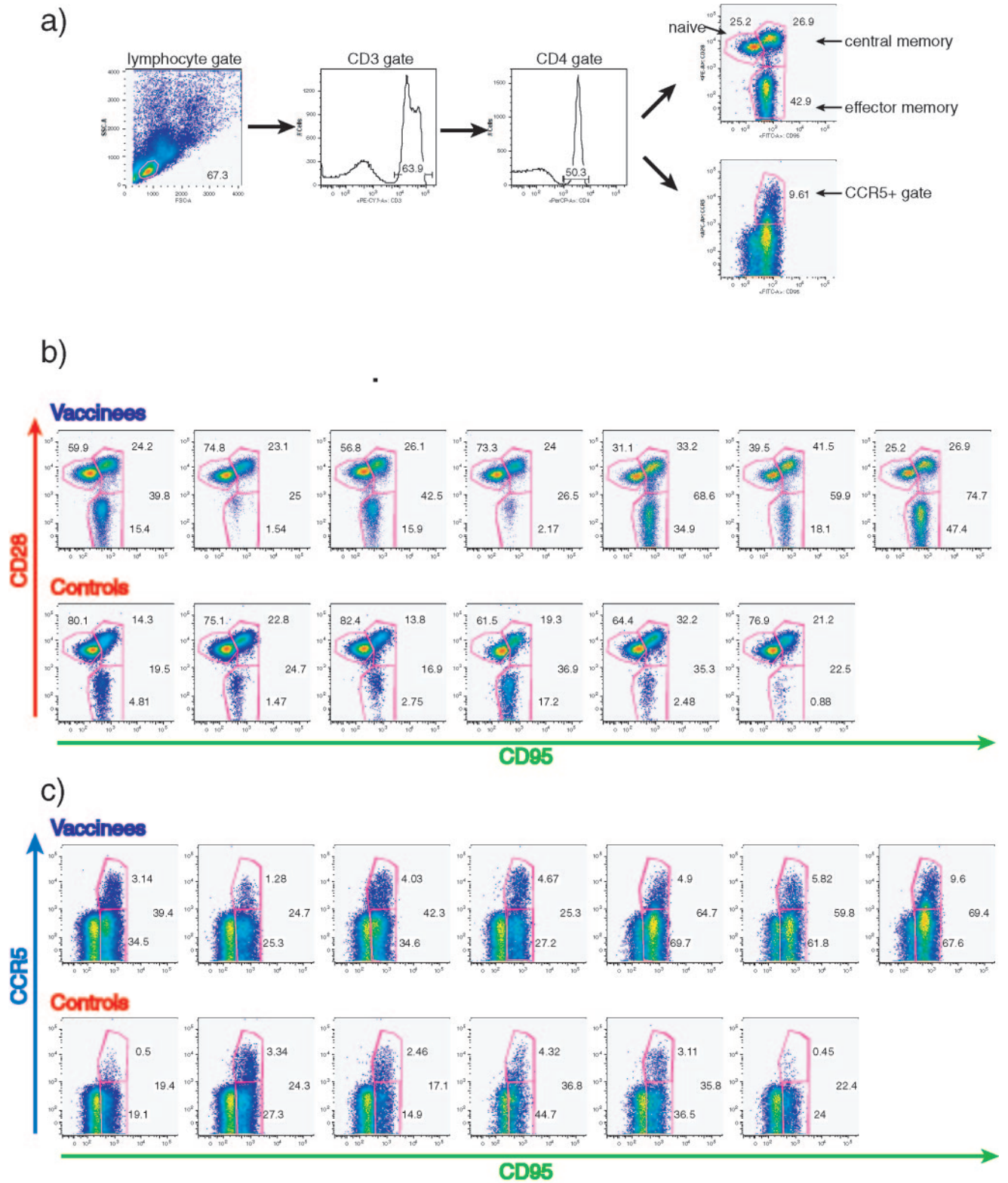

FIG. 5. Flow analysis of $CD4^+$ populations. PBMC were stained with antibodies to CD3, CD4, CD28, CCR5, and CD95 and read on an LSR II flow cytometer. (a) Cells were first gated through a lymphocyte gate and then gated through $CD3^+$ and $CD4^+$ gates. The resultant population was then gated for CD28 versus CD95 or CCR5 versus CD95, and populations were labeled as indicated. Cells are gated through a lymphocyte gate by forward and side scatter and then gated subsequently for $CD3^+$ and $CD4^+$ populations. (b) Effector memory is defined as the population of cells in the CD95 versus CD28 dot plot as being $CD95^+$ $CD28^-$. Central memory is $CD95^+$ $CD28^+$, and naive $CD4^+$ T cells are $CD95^-$ $CD28^+$. (c) $CCR5^+$ memory cells are $CCR5^+$ and $CD95^+$. FITC, fluorescein isothiocyanate.

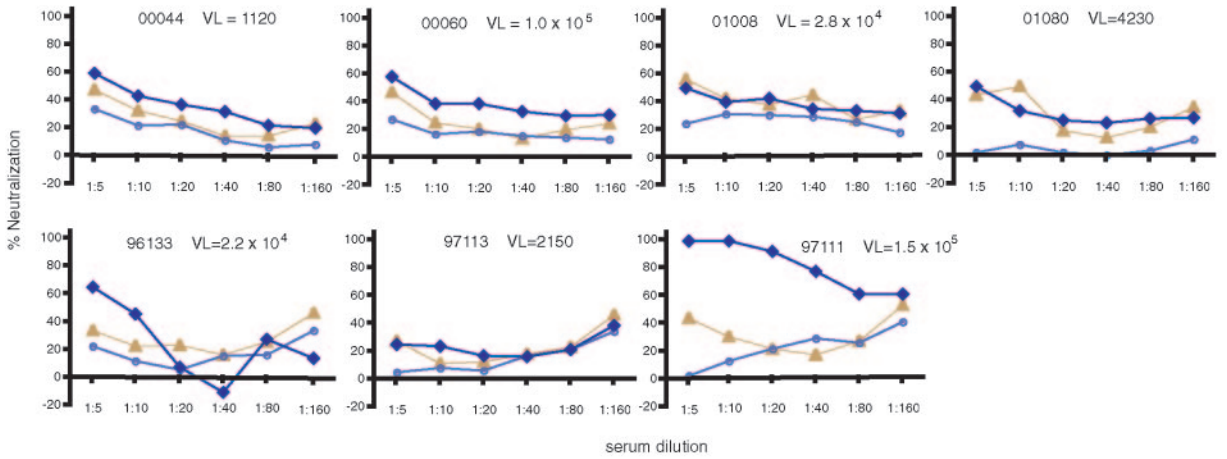

FIG. 6. Six of seven vaccinees did not develop neutralizing antibodies by days 170 to 210 postinfection. Neutralizing antibodies were analyzed using pseudovirions with the SIVmac239 envelope, which is a more sensitive test than by infection of PBMC with wild-type virus (68). The pseudovirus contains luciferase, and results indicate how much the luciferase signal is decreased by neutralizing antibodies present in the plasma. Animal numbers and viral loads (VL) at the time of the assay are indicated. A comparison of individual viral loads and the geometric mean for each group are shown in Fig. 3. Results are displayed as percentages of neutralization. Prevaccination assays are indicated by tan triangles, prechallenge assays are denoted by light blue circles, and postinfection results are displayed as a blue diamonds.

ited preservation of the memory CD4 subset, did not have significant titers of neutralizing antibodies. Several lines of evidence implicate immunodominant $CD8^+$ lymphocyte responses in control. In the previous study, using only Gag in a DNA prime-Ad5 boost regimen, there was no evidence for control in Mamu-A*01-negative macaques (11). Interestingly, *Mamu-A*01*-positive macaques showed some level of control, if only temporarily, and this control might be attributable to the Gag $CM9_{181–189}$-specific $CD8^+$ responses present in these animal. It is, therefore, possible that induction of Gag $CM9_{181–189}$-specific $CD8^+$ responses played a large role in control of viral replication in our vaccinees. This might have been aided by vaccine-induced immune responses to Tat $SL8_{28–35}$, another immunodominant T-cell response in *Mamu-A*01*$^+$ macaques. Indeed, these two $CD8^+$ responses dominated the acute-phase anamnestic $CD8^+$ responses, comprising more than 50% of anamnestic responses (Fig. 2). This concept could be tested by removing the immunodominant epitopes from both the vaccine and the challenge virus in subsequent experiments. Additionally, despite their immunodominance, these two responses do not show a rank correlation with either decreased peak viremia or decreased set point viremia. Finally, Tat $SL8_{28–35}$ escapes rapidly, and by week 16 postinfection, all infected animals showed escape in this epitope, but vaccinees maintained control of viremia long after week 16 (data not shown).

While these experiments show the best observed non-Env vaccine-mediated control of SIVmac239 replication in Indian rhesus macaques thus far, there are several caveats. First, all

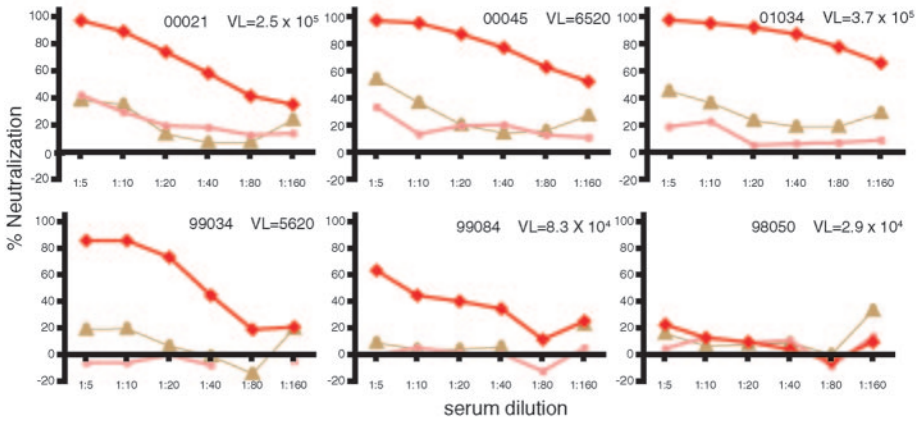

FIG. 7. Five of six control animals developed neutralizing antibodies by day 170 to 210 postinfection. Neutralizing antibodies were analyzed using pseudovirions with the SIVmac239 envelope, which is a more sensitive test than by infection of PBMC with wild-type virus (68). The pseudovirus contains luciferase, and results indicate how much the luciferase signal is decreased by neutralizing antibodies present in the plasma. Animal numbers and viral loads (VL) at the time of the assay are indicated. A comparison of individual viral loads and the geometric mean for each group are shown in Fig. 3. Results are displayed as percentages of neutralization. Prevaccination assays are indicated by tan triangles, prechallenge assays are denoted by light red circles, and postinfection results are displayed as red diamonds.

eight vaccinees were *Mamu-A*01*$^+$, and in the *gag*-only study, it was observed that animals expressing this allele do better after challenge, especially if they are vaccinated with regions of the virus that contain targets of their immunodominant CD8$^+$ responses. Thus, we need to test this vaccine regimen in *Mamu-A*01*$^-$ macaques. Second, the challenge virus strain was exactly matched to the viral sequences in the vaccine. We therefore need to use a heterologous challenge to determine whether this regimen might have a chance of being effective in the field. Finally, the repeated low-dose challenge was administered 6 months after the Ad5 boost. We do not know enough about the long-term evolution of vaccine-induced memory cells to predict how long this vaccine-induced memory response will remain efficacious.

Despite these significant caveats, this study indicates that vaccine-induced cellular immune responses can control replication of a highly pathogenic SIV to a significant degree. The results raise the possibility that a vaccine inducing only cellular responses has the potential to contribute to control of SIVmac239.

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